

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.

B9



Europäisches Patentamt

European Patent Office

Office européen des brevets

Publication number:

0 256 714

A2



EUROPEAN PATENT APPLICATION

(1) Application number: 87306753.2

(2) Int. Cl. A61K 39/395
(A61K39/395.37:02)

(3) Date of filing: 30.07.87

(4) Priority: 01.08.86 US 892596
29.05.87 US 55681

(5) Applicant: CETUS CORPORATION
1400 Fifty-Third Street
Emeryville California 94608(US)

(6) Date of publication of application:
24.02.88 Bulletin 88/08

(7) Inventor: Stevens, Paul
1126 El Centro Avenue
Oakland California 94602(US)
Inventor: Houston, L L
1901 Oak Crest Drive
Oakland California 94602(US)
Inventor: Zimmerman, Robert
3912 N. Peardale Drive
Lafayette California 94549(US)
Inventor: Koths, Kirston Edward
2646 Mira Vista Drive
El Cerrito California 94530(US)
Inventor: Issell, Brian
255 Fernwood Drive
Moraga California 94556(US)

(8) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(9) Representative: Blizley, Richard Edward et al
BOULT, WADE & TENNANT 27 Fenchurch Street
London EC4A 1PO(GB)

(10) Combination therapy using anti-tumor monoclonal antibodies and/or immunotoxins with interleukin-2.

(11) Anti-tumor activity in humans can be augmented by administering to the mammalian host a pharmacologically effective amount of mammalian IL-2 and at least one immunotoxin that binds selectively to human tumor cells and/or at least one monoclonal antibody that binds selectively to human tumor cells. The IL-2 and immunotoxin and/or antibody are preferably administered separately to the host. The compositions and medications of the invention are useful for prophylactic or therapeutic treatment of such cancers as ovarian and

EP 0 256 714 A2

0 256 714

COMBINATION THERAPY USING ANTI-TUMOR MONOCLONAL ANTIBODIES AND/OR IMMUNOTOXINS
WITH INTERLEUKIN-2

This invention relates to use of a combination of interleukin-2 and at least one monoclonal antibody and/or immunotoxin directed against tumor cells in the therapeutic or prophylactic antitumor treatment of mammals using simultaneous or alternate administration of these components.

Interleukin-2 (IL-2), a lymphokine produced by normal peripheral blood lymphocytes that induces proliferation of antigen or mitogen stimulated T cells after exposure to plant lectins, antigens, or other stimuli, was first described by Morgan, D. A., et al., Science (1976), 193:1007-1008. Then called T cell growth factor because of its ability to induce proliferation of stimulated T lymphocytes, now interleukin-2 is recognized as modulating a variety of functions of immune system cells *in vitro* and *in vivo*.

IL-2 was initially made by cultivating human peripheral blood lymphocytes (PBL) or other IL-2-producing cell lines. See, for example, U.S. Patent No. 4,401,756. Recombinant DNA technology has provided an alternative to PBLs and cell lines for producing IL-2. Taniguchi, T. et al., Nature (1983), 302:305-310 and Devos, R., Nucleic Acids Research (1983), 11:4307-4323 have reported cloning the human IL-2 gene and expressing it in microorganisms.

U.S. Patent No. 4,518,584 describes and claims muteins of IL-2 in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been replaced with a neutral amino acid, such as serine or alanine. U.S. Patent Nos. 4,530,787 and 4,569,790 disclose and claim methods for purifying recombinant native IL-2 and muteins thereof, as well as the purified form of IL-2.

U.S. Patent No. 4,604,377 issued August 5, 1986 discloses an IL-2 composition suitable for reconstituting in a pharmaceutically acceptable aqueous vehicle composed of oxidized microbially produced recombinant IL-2. The IL-2 is noted as useful in combination with cytotoxic chemotherapy or irradiation or surgery in the treatment of malignant or pre-malignant diseases in a direct therapeutic or adjuvant setting or in combination with other immune-modulating drugs, lymphokines (e.g., IL-1, IL-3, CSF-1 and IFNs), or naturally occurring or inducible anti-cellular toxins.

Rosenberg and his coworkers have shown that systemic administration of recombinant IL-2 in high doses causes regression of established metastatic cancers in mice (Rosenberg et al., J. Exp. Med. (1985) 161:1169-1188); and, in conjunction with lymphokine-activated killer cells (Rosenberg, S. et al., New Eng. J. Med. (1985), 313:1485-1492), and tumor-infiltrating lymphocytes (Rosenberg et al., Science (1986) 233:1318-1321), in humans.

Since the mid-1970s, there have been numerous reports of murine monoclonal antibodies that interact with human breast cancer associated antigens. In these reported studies, mice were immunized and boosted with human milk fat globule proteins, breast cancer cell lines or breast cancer membrane extracts. Immune splenocytes were fused with mouse myeloma cells and hybridomas were selected based on some specificity of the culture supernatant for breast or breast cancer antigens. Taylor-Papadimitriou, J. et al., Int. J. Cancer (1981) 28:17-21; Yuan, D., et al., JNCI (1982) 68:719-728; Ciocca, D. R. et al., Cancer Res. (1982) 42:4256-4258.

More recently, investigators at Cetus Corporation have discovered murine monoclonal antibodies that bind selectively to human breast cancer cells, are IgGs or IgMs, and, when conjugated to ricin A chain to form an immunotoxin, exhibit a tissue culture inhibitory dose which results in 50% of control (untreated) protein synthesis (TCID 50%) at immunotoxin concentrations of less than about 10nM against at least one of MCF-7, CAMA-1, SKBR-3, or BT-20 cells. These antibodies are described more fully in EPC Patent Publication No. 153,114 published August 28, 1985.

In addition, researchers at Cetus Corporation have discovered murine monoclonal antibodies which do not bind to blood cells, have a breast tumor binding range of at least 0.25 (i.e., they bind to at least 25% of breast tumors tested) or have a breast cancer cell line binding range of greater than or equal to 0.25, have a normal tissue reactivity as defined below for human breast and/or ovarian cells equal to or less than 0.09, are IgGs or IgMs, and, when conjugated to an imaging moiety, produce a signal sufficient to image breast cancer tumors. These antibodies include most of those described above and are described more fully in European Pat. Pub. No. 220,858 published May 6, 1987.

Immunotoxins, which are comprised of an antibody conjugated to a toxin, have been used for therapy of various cancers to which the antibody is specific. Certain immunotoxin molecules may be too large to reach the tumor cells efficiently due to poor diffusion out of capillaries.

0 256 714

Combination chemotherapy using two or more anti-cancer drugs to treat malignant tumors in humans is currently in use in research and in the clinic. The anti-cancer drugs may be antimetabolites, alkylating agents, antibiotics, general poisons etc. Combinations of drugs are administered in an attempt to obtain a synergistic cytotoxic effect on most cancers, e.g., carcinomas, melanomas, lymphomas and sarcomas, and to reduce or eliminate emergence of drug-resistant cells and to reduce side effects of each drug.

Dr. Rosenstein et al. in Immunol. (1986) 137:1735-1742 disclosed that IL-2 increases the vascular permeability and rate of serum albumin diffusion into organs. Itoiz et al. in J. Immunol. (1985) 135:2965 disclosed that reversible fluid retention problems result from IL-2 administration.

To applicants' knowledge no one has administered immunotoxin and/or monoclonal antibody and IL-2 to decrease or eliminate tumor burden.

Accordingly, the present invention provides a composition suitable for parenteral or subcutaneous administration to a warm-blooded mammalian host for therapeutic or prophylactic treatment of tumor burden comprising a mixture, in pharmacologically effective amounts, of IL-2 from a mammalian species and at least one monoclonal antibody and/or immunotoxin that binds selectively to cells containing the tumor burden.

In another aspect, the invention provides a method for therapeutic or prophylactic treatment of tumor burden in a warm-blooded mammalian host comprising administering to said host a pharmacologically effective amount of a combination of IL-2 from a mammalian species and at least one monoclonal antibody and/or immunotoxin that binds selectively to cells containing the tumor burden.

Preferably the IL-2 is a recombinant human IL-2, and the monoclonal antibody employed alone and/or in the immunotoxin selectively binds to human breast and/or ovarian cancer cells and has a G or M isotype, and the tumor burden line treated is breast and/or ovarian cancer.

The combination of IL-2 immunotoxin(s) and/or antibodies) in pharmacologically effective amounts is expected to provide suitable treatment of a variety of forms of cancer, especially breast and ovarian cancers.

The invention includes the use of a combination of IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human tumor cells and/or at least one immunotoxin that binds selectively to human tumor cells in providing a medication for the therapeutic or prophylactic treatment of tumor burden in a warm-blooded mammalian host.

Preferably, the IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human tumor cells and/or at least one immunotoxin that binds selectively to human tumor cells are to be administered separately.

In yet another aspect, the invention includes a composition suitable for parenteral or subcutaneous prophylactic treatment of tumor burden comprising a mixture, in pharmacologically effective amounts, of IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human tumor cells.

A further aspect of the invention is IL-2 from a mammalian species and at least one immunotoxin that binds selectively to cells containing the tumor burden and/or at least one monoclonal antibody that binds selectively to human tumor cells for use in a method for therapeutic or prophylactic treatment of tumor burden in a warm-blooded mammalian host comprising administering to said host a pharmacologically effective amount of a combination of said IL-2, immunotoxin(s) and/or antibodies; optionally with the components of said combination to be administered separately to the host.

The invention also includes a process for the preparation of a composition suitable for parenteral or subcutaneous administration to mammalian hosts for therapeutic or prophylactic treatment of cancer comprising mixing together or formulating for such use IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human tumor cells and/or at least one immunotoxin that binds selectively to human tumor cells.

The term "therapeutic" treatment refers to administration to the mammalian host or patient of the IL-2 and immunotoxin(s) after the patient has developed cancer, (i.e., after a tumor burden has been determined), as measured by any means in the art, with resultant decrease or elimination of the existing tumor burden.

The term "prophylactic" treatment refers to such administration to prevent recurrence of the cancer after therapeutic treatment has been administered.

The terms "cancer" and "tumor burden" refer to any neoplastic disorder, including such cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, breast cancer, sarcoma, prostate, pancreatic endometrial, and ovarian carcinomas, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancer and gastrointestinal or stomach cancer. In the method of the invention, the target tumor burden is advantageously breast and/or ovarian cancer.

The term "pharmacologically effective amount" refers to the total amount of each active component of the method or composition herein that is sufficient in showing a meaningful patient benefit, i.e., prolongation of life and/or reduction of disease. When the effective amounts defined herein are employed, more efficacy is obtained using the combination than using either component alone. As applied to an individual active ingredient administered alone, the term refers to that ingredient alone; when combinations are used, the term refers to combined amounts in the preparation that result in the therapeutic or prophylactic effect.

The term "recombinant" refers to IL-2 produced by recombinant DNA techniques wherein generally the gene coding for the IL-2 is cloned by known recombinant DNA technology. For example, the human IL-2 gene is inserted into a suitable DNA vector such as a bacterial plasmid, preferably an *E. coli* plasmid, to obtain a recombinant plasmid, and the plasmid is used to transform a suitable host. The gene is expressed in the host to produce the recombinant protein. Examples of suitable recombinant plasmids for this purpose include pBR322, pCR1, pMB9 and pSC1. The transformed host may be prokaryotic or eukaryotic, including mammalian, yeast, *Aspergillus* and insect cells. One preferred embodiment herein, but not the only preferred embodiment, employs bacterial cells as the host.

The term "pharmaceutically acceptable" refers to a carrier medium that does not interfere with the effectiveness of the biological activity of the active ingredients and that is not toxic to the hosts to which it is administered.

As used herein, the term "monoclonal antibody" means an antibody composition having a homogeneous antibody population. It is not intended to be limited as regards the source of the antibody or the manner in which it is made.

The term "immunotoxin" as used herein refers to a conjugate of an antibody or fragment of an antibody and a cytotoxic moiety. The antibody or fragment thereof employed must bind selectively to human tumor cells and be effective in an immunotoxin. The antibody is chosen from those described hereinafter if it is effective in the conjugated form. The cytotoxic moiety of the immunotoxin includes a cytotoxic drug or an enzymatically active toxin of bacterial or plant origin or an enzymatically active fragment ("A chain") of such a toxin. Examples of enzymatically active toxins and fragments thereof include diphtheria A chain, nonbinding fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, and enomycin. Ricin A chain, nonbinding active fragments of diphtheria toxin, abrin A chain, and PAPII are preferred. Most preferred is the ricin A chain.

As used herein, the term "selective binding to human tumor cells" refers to preferential binding of the antibodies of the immunotoxin to human cells that are cancerous or exhibit cancerous growth or other properties characteristic of cancer. The antibodies of the immunotoxins do not preferentially bind to normal healthy cells. Examples of such tumor cells include leukemia cells, prostate cancer cells, colorectal cancer cells, breast cancer cells, ovarian cancer cells, rectal cancer cells, throat cancer cells, melanoma cells, colon cancer cells, bladder cancer cells, lung cancer cells, and gastrointestinal or stomach cancer cells. Most preferably, the antibodies of the immunotoxins selectively bind to breast and/or ovarian cancer cells, as opposed to binding to normal, non-cancerous cells.

As used herein with respect to the exemplified monoclonal anti-human breast cancer antibodies of the immunotoxins, the term "functional equivalent" means a monoclonal antibody that: (a) has a breast tumor binding range of at least 0.25 or has a breast cancer cell line range of greater than or equal to 0.25; (b) binds selectively to human breast cancer cells; (c) has a G or M isotype; and (d) binds to the same antigen or epitope as the exemplified monoclonal antibody, as determined by immunoprecipitation or crossblocking and sandwich immunoassay.

As described above, the term "functional equivalent" as used herein includes four criteria. The last of these criteria, binding to the same antigen or epitope as an exemplified monoclonal antibody, may be demonstrated by experiments which show crossblocking of an exemplified monoclonal antibody by the functionally equivalent monoclonal antibody. Crossblocking occurs as a result of an antibody binding to the same epitope on an antigen as that bound by one of the exemplified antibodies, or as a result of an antibody binding to a different epitope which is so closely situated on the same antigen that binding of an antibody to one epitope blocks the binding of an antibody to the second epitope. Crossblocking thus is one of the criteria by which one can determine that a functionally equivalent monoclonal antibody binds to the same antigen or epitope as an exemplified monoclonal antibody.

0 256 714

So-called "sandwich" assays are another method for determining whether an antibody of the immunotoxin binds the same antigen or epitope. In these assays, a first monoclonal antibody is bound to a support, for example, the surface of a microtiter plate well. After treatment to prevent nonspecific binding, a soluble antigen preparation is added to the bound antibody. Subsequently, a second antibody, having a detectable label, for example, a chromogenic enzyme is added. If the second antibody binds to the antigen, a different epitope specificity or multiple copies of the same epitope on the same antigen are indicated. If the second antibody fails to bind, either the same epitope specificity or different but proximal antigen specificity is indicated. The results of both the crossblocking and sandwich assay are further defined by a second series of tests such as immune precipitation or Western blotting to characterize the molecular weight(s) of the antigen(s) bound by both antibodies.

The American Type Culture Collection (ATCC), Rockville, MD USA has a wide variety of cell lines on deposit which produce monoclonal antibodies to target tumors. For example, cell lines producing monoclonal antibodies to human non-small cell lung cancer include 703D4 (deposited as ATCC No. HB8310). Cell lines producing monoclonal antibodies to human melanoma cells include 704A1 (deposited as ATCC No. HB8302). Cell lines producing monoclonal antibodies to small cell carcinoma include the cell lines deposited as ATCC HB8462 and ATCC HB8711. Cell lines producing antibodies to pancreatic carcinoma of ductal origin include the hybridoma deposited as ATCC HB8504. A cell line producing antibodies which bind to an epitope present on adenocarcinomas of the stomach, colon, and pancreas, and to esophagus, breast and ovarian tumors, known as CSLEX1, is deposited as ATCC HB8580.

Conjugates of the antibody and cytotoxic moiety may be made using a variety of bifunctional protein modifying reagents. Examples of such reagents include N-succinimidyl-3-(2-pyridyl)dithio) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters such as dimethyl adipimidate • HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis-(p-azidobenzoyl)-hexanediamine, bis-diazonium derivatives such as bis-(p-diazonium-benzoyl)-ethylenediamine, diisocyanates such as tolylene-2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene.

The method of this invention involves administering to a warm-blooded mammalian host, including a mouse, rat, rabbit, primate, pig or human host, preferably a human patient, a pharmacologically effective amount of IL-2 and one or more monoclonal antibody(ies) and/or immunotoxins that selectively bind to human tumor cells. The IL-2 and monoclonal antibody(ies) and/or immunotoxin(s) may be combined in vitro before administration if neither is adversely affected chemically and both remain efficacious. Preferably, however, they are separately administered to the patient, in either order or simultaneously. An example is the protocol set forth in Examples 1 and 2 hereof, wherein the IL-2 and immunotoxin are administered separately.

The administration(s) may take place by any suitable technique, including parenteral administration. Examples of parenteral administration include intravenous, intraarterial, intramuscular, subcutaneous, and intraperitoneal, with intravenous, intramuscular and intraperitoneal administration being preferred.

As an example, the patient/host may be treated locally (as by peritumor or intramuscular injection) or systemically with a preparation having IL-2 activity until the capillaries begin to leak large proteins (about 6 days). Then the immunotoxin may be administered with or without the IL-2 for the prescribed duration of treatment. Alternatively, immunotoxin can be administered from day 1 of treatment. Local treatment with IL-2 to promote immunotoxin action may be followed with systemic (e.g., intraperitoneal or intravenous bolus) administration of IL-2 and immunotoxin.

The dose and dosage regimen will depend on whether the IL-2 and antibody(ies) and/or immunotoxin(s) are being administered separately or as a mixture, the type of antibody(ies) and/or immunotoxin(s) and cancer, the patient/host and the patient's history. The amount must be effective to achieve some tumor reduction or augmentation of LAK activity. The doses may be single doses or multiple doses. If multiple doses are employed, as preferred, the frequency of administration will depend, for example, on the type of component, cancer, dosage amounts, host, etc. For some types of cancers, daily administration may be effective, whereas for other types of cancer, administration every other day or every third day may be effective, but daily administration ineffective. The practitioner will be able to ascertain from clinical trials which route of administration and frequency of administration are most effective in humans in any particular case.

The dosage amount which appears to be most effective herein is one which results in regression in size of the tumor or complete disappearance or non-reappearance of the tumor, and is not toxic or is acceptably toxic to the host. Generally, such conditions as fever, chills and general malaise are considered acceptable. This optimum dose level will depend on many factors, for example, on the type of host and type of cancer, route, schedule and sequence of administration, existing tumor burden, the type of IL-2 and antibody(ies) and/or immunotoxin(s), and the definition of toxicity.

Toxicity to the host may be defined by the extent and type of side effects, with fever, chills and general malaise considered acceptable toxicity for the study herein, or sometimes by the amount of body weight gain or by death after a certain period of time. The reversible fluid retention in the body resulting from IL-2 administration is disclosed by Lotze, et al., *J. Immunol.*, 135:2865 (1985). If body weight gain is the criterion for toxicity, typically, a gain of from 10 to 20% by weight will be tolerated, with greater than 20% gain being considered toxic.

If there is acceptable toxicity, if the host is immunocompetent, and if the route of administration is pre-IL-2 treatment and/or concurrent administration of IL-2 daily for 14 days beginning at day 1 post-treatment and/or antibody(ies) every other day or every third day beginning at day 1, the dosage level for each administration of recombinant, microbially produced IL-2 and an anti-breast cancer monoclonal antibody is preferably about 3.375×10^4 - 7.5×10^5 units (U) IL-2 per kg of host weight (where 3000 units is 1 μ g), based on maximum tolerated dose studies, and from about 25 to 50 mg/kg of host weight of antibody.

If there is acceptable toxicity, and if there is pretreatment with recombinant, microbially produced IL-2 and/or concurrent administration daily for 14 days of the IL-2 and for 7 days of immunotoxin beginning at day 1 post-treatment, the dosage level of each administration of immunotoxin made with the anti-breast cancer antibody is 25 to 500 μ g/kg of host weight of immunotoxin. The IL-2 level is that given above. If the host is immuno-compromised (i.e., nude mice, which have a genetic deficiency), the maximum tolerated dose may be lower.

In one preferred embodiment the IL-2 is given at the maximum tolerated dose daily for one week followed by giving one-half the maximum tolerated dose of IL-2 with concomitant administration of the maximum tolerated dose of antibody or immunotoxin.

For parenteral administration the IL-2 and antibody(ies) and/or immunotoxin(s) will generally each be formulated in a unit dosage injectable form (solution, suspension, emulsion), preferably in a pharmaceutically acceptable carrier medium that is inherently non-toxic and non-therapeutic. Examples of such vehicles include saline, Ringer's solution, dextrose solution, mannitol, and normal serum albumin. Non-aqueous vehicles such as fixed oils and ethyl oleate may also be used. The carrier medium may contain minor amounts of additives such as substances that enhance isotonicity, solubility, and/or chemical stability, e.g., buffers, detergents, and preservatives. The IL-2 and antibody(ies) and/or immunotoxin(s) will typically each be formulated in such carriers at a concentration of about 0.1mg/ml to 100mg/ml, preferably 0.2 to 1mg/ml.

Alternatively, the IL-2 and antibody(ies) and/or immunotoxin(s) may be made into a sterile, stable lyophilized formulation in which the purified IL-2 and antibody(ies) and/or immunotoxin(s) are admixed with a water-soluble carrier such as mannitol, which provides bulk, and about 500 μ g of a surfactant such as sodium dodecyl sulfate per mg of IL-2 or 0.01-0.05% in typical formulations to ensure the solubility of the recombinant IL-2 in water, if the antibody/immunotoxin is still active at such concentrations. The formulation is suitable for reconstitution in aqueous injections for parenteral administration and it is stable and well-tolerated in the mammalian host, particularly in human patients. The IL-2 formulation method is more completely described in U.S. Patent No. 4,604,377 issued August 5, 1986.

In an alternative IL-2 formulation of PCT Publication No. WO87/00056, published January 15, 1987, the IL-2 may be solubilized, not by a detergent, but by reacting the IL-2 with an activated polymer selected from polyethylene glycol homopolymers and polyoxyethylated polyols, said polymer having a molecular weight of from 300 to 100,000 daltons, preferably 350 to 40,000 daltons. The polymer is activated terminal groups reactive with both the free amino or thiol groups of the IL-2 and the hydroxyl group of the polymer. Examples of such coupling agents include hydroxynitrobenzene sulfonic ester, cyanuric acid chloride, and N-hydroxysuccinimide. This modification eliminates the necessity of adding detergents to solubilize the IL-2 at physiological pH. The IL-2 is then formulated directly with the water-soluble carrier and buffer as described above, and the formulation may be lyophilized and the lyophilized mixture reconstituted as described above.

As mentioned above, it is preferred not to admix the components, but rather to administer them separately. If the formulation contains two or more of the components, the relative amounts of each may vary within the ranges described above depending on the efficacy obtained.

0 256 714

The IL-2 herein may be any IL-2 prepared from tissue cultures or by recombinant techniques, and from any mammalian source such as, e.g., mouse, rat, rabbit, primate, pig, and human. Preferably the IL-2 is from a human source. More preferably the IL-2 is recombinant.

The recombinant IL-2 may be obtained as described by Taniguchi et al., Nature, 302:305-310 (1983) and Devos, Nucleic Acids Research, 11:4307-4323 (1983), by cloning the native human IL-2 gene and expressing it in transformed microorganisms. It may also be an IL-2 mutein as described in U.S. Patent No. 4,518,584, in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been replaced by a neutral amino acid such as serine or alanine or an IL-2 mutein in which the methionine normally occurring at position 104 of the wild-type or native molecule has been replaced by a neutral amino acid such as alanine.

In one embodiment the IL-2 is an unglycosylated protein which is produced by a microorganism which has been transformed with the human cDNA sequence or a modified human cDNA sequence of IL-2 which encodes a protein with an amino acid sequence at least substantially identical to the amino acid sequence of native human IL-2, including the ability to form the disulfide bond between the cysteines at positions 58 and 105, and has biological activities which are common to native human IL-2. The IL-2 may also be produced from yeast or other hosts, as described above. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) which do not cause an adverse functional dissimilarity between the synthetic protein and native human IL-2. Examples of IL-2 proteins with such properties include those described by Taniguchi et al., Nature (1983), 302:305-310; Devos, Nucleic Acids Research (1983), 11:4307-4323; and by European Patent Publication Nos. 91,539 and 88,195; in U.S. Patent 4,518,584, supra. Most preferably, the IL-2 is the des-alanine-IL-2₋₁₂₅ mutein in which the N-terminal alanine of the native IL-2 is deleted and the cysteine at position 125 of the native IL-2 is replaced by a serine residue, the des-alanine-IL-2₋₁₀₄-serine-125 mutein in which the methionine at position 104 of the native IL-2 is replaced by an alanine residue and the cysteine at position 125 is replaced by a serine residue, or IL-2 wherein any combination of up to five of the first five N-terminal amino acid residues are deleted.

IL-2 may be produced and purified to clinical purity by the method described and claimed in U.S. Patent No. 4,569,790, issued February 11, 1985.

The antibodies useful herein are produced from hybridomas prepared from antibody-producing fusion partners. Such fusion partners are generated by immunizing mice with live human cancer cells, such as breast cancer cells, or membrane extracts made therefrom. The mice are inoculated intraperitoneally with an immunogenic amount of the cells or extract and then boosted with similar amounts of the immunogen. Spleens are collected from the immunized mice a few days after the final boost and a cell suspension is prepared therefrom for use in the fusion.

Hybridomas are prepared from the splenocytes and a murine tumor partner using the general somatic cell hybridization technique of B. Kohler and C. Milstein, Nature (1975) 256:495-497 as modified by Buck, D. W. et al., In Vitro (1982) 18:377-381. Available murine myeloma lines, such as those from the Salk Institute, Cell Distribution Center, San Diego, California, USA, may be used in the hybridization. Basically, the technique involves fusing the tumor cells and splenocytes using a fusogen such as polyethylene glycol. After the fusion the cells are separated from the fusion medium and grown in a selective growth medium, such as HAT medium, to eliminate unhybridized parent cells. The hybridomas are expanded, if desired, and supernatants are assayed for anti-human cancer activity by conventional immunoassay procedures (e.g., radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay) using the immunizing agent (cancer cells or membrane extract) as antigen. Positive clones are characterized further to determine whether they meet the criteria of the antibodies herein, i.e., whether they selectively bind to human tumor cells.

Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, as the case may be, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired.

The preferred monoclonal antibodies (e.g. for the immunotoxins) herein bind selectively to human breast and/or ovarian cancer cells, and, therefore, such cells are used as immunizing agent in the above-described protocol.

The important characteristics of the preferred monoclonal antibodies (e.g. for the immunotoxins) are (1) their immunoglobulin class, (2) their selectivity for human breast and/or ovarian cancer cells, (3) the range of human breast cancer cell lines to which they bind, and (4) the range of human breast tumor sections to which they bind, and (5) their ability to form an active immunotoxin.

The selectivity and range of a given preferred antibody (e.g. for the immunotoxin) is determined by testing it against panels of (1) human breast cancer tissues and cells and (2) normal human tissues or cells of breast or other origin. In selecting the preferred class of antibodies, approximately 22,000 growing hybridoma cultures were initially screened against the immunizing breast tumor membranes or cell line. A panel of seven normal tissue membranes, a fibroblast cell line, and a breast tumor frozen section Clones that reacted with the neoplastic materials, but not with the normal materials, were identified in this initial screen and chosen for isotyping and additional screening for selectivity and range. The additional screening involved sixteen normal tissue sections, five normal blood cell types, eleven non-breast neoplasm sections, twenty-one breast cancer sections, and fourteen breast cancer cell lines.

For the preferred antibodies (e.g. for the immunotoxins), the words "specificity" and "normal tissue reactivity" are used interchangeably and are defined as the sum of the number of substructures stained in sixteen normal tissue frozen sections and the number of blood cell types bound, divided by the sum of the total number of substructures bound by any of the monoclonal antibodies in all the tissue on which the monoclonal antibodies were tested and five blood cell types tested.

The term "tumor range" is defined as the number of breast tumor frozen sections stained divided by the number of breast tumor frozen sections tested. The term breast cancer "cell line range" is defined as the number of breast cancer cell lines stained divided by the number of breast cancer cell lines tested. The antibodies of the immunotoxins herein preferably have a normal tissue reactivity equal to or less than 0.09, and a breast tumor binding range of equal to or greater than 0.25 or a breast cancer cell line binding range of equal to or greater than 0.25.

Antibodies of five of the thirty-three deposited hybridomas herein were found to recognize the same 200 K dalton antigen. Antibodies of four of the thirty-three bound to a 240 K dalton intracellular antigen. Three bound to one or more high molecular weight mucins (HMW) and two bound to transferrin receptors in the form of a 95 K dalton antigen. All antigen weights mentioned herein were determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis under reducing conditions using procedures known in the art.

The immunotoxins herein may be prepared by conjugating a toxin as described above, such as ricin A chain, to one of the above-described antibodies using the coupling agents defined above. The technique for preparing such immunotoxins is described in EP Publication No. 153,114, published February 8, 1985.

The following examples provide a detailed description of the preparation and characterization of representative monoclonal antibodies for conjugation as well as an immunotoxin for use with IL-2 in accordance with this invention. These examples are not intended to limit the invention in any manner. In the examples, all parts and percentages for solids are given by weight-weight unless otherwise indicated, and all parts and percentages for liquids are given by volume/volume unless otherwise indicated.

35

Example 1 A

Antibody Characterization

40

Fresh post-surgical human breast cancer tissue and a variety of normal tissues were used to prepare membrane extracts by homogenization and discontinuous sucrose gradient centrifugation. Human breast cancer cell lines were obtained from the Breast Cancer Task Force, from the American Type Culture Collection (ATCC), and from Dr. Jorgen Fogh at Memorial Sloan Kettering. The cells were maintained and passaged as recommended by the Breast Cancer Task Force, the ATCC, and Dr. Fogh. For immunizations, either membrane extract containing 100 µg of protein (Lowry assay) or ten million live breast cancer cells were inoculated intraperitoneally into five-week-old Balb/c mice. The mice were boosted identically twice at monthly intervals. Three days after the last boost, the spleens were removed for cell fusion.

50

Hybridoma Methods

55

Somatic cell hybrids were prepared by the method of Buck, D. W., et al, supra, using the murine myeloma line Sp-2/0/Ag14. All hybridoma cell lines were cloned by limiting dilution. Half of the fusions employed splenocytes from mice immunized with breast cancer membrane extracts and half used splenocytes from mice immunized with live breast cancer cell lines. Eighty-three thousand four hundred twenty-four wells were generated from those fusions, of which 22,459 exhibited hybridoma growth.

0 256 714

Screening Methods

Hybridoma supernatant was assayed for reactive antibody in either a solid phase enzyme-linked immunosorbent assay (ELISA) with the immunizing breast cancer membrane extract or an indirect immunofluorescence assay with the immunizing breast cancer cell line. For the solid phase membrane ELISA, 40 μ l of 0.1 mg/ml breast cancer membrane protein were placed in polyvinyl chloride (PVC) microtiter wells for 12 hours at 4°C. The extract was aspirated and the wells were washed with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). The wells were then incubated with 45 μ l of a 1:10 dilution of hybridoma supernatant. The diluent was medium with 25 mM of a buffer, 10% bovine serum, and 0.1% sodium azide. After 30 minutes at room temperature, the wells were again washed and incubated 45 minutes at 37°C with a 1:200 dilution of peroxidase conjugated goat anti-mouse IgG. The diluent was PBS. The wells were then washed with PBS and reacted with 200 μ l of 1,2-azinobi(3-ethylbenzothiazoline sulphonic acid) in 0.1 M sodium citrate buffer pH 4.2 for 30 minutes at room temperature. Optical density was measured at 405 nm. For each experiment, a positive control, anti-beta 2 microglobulin at 5 μ g/ml, was reacted with normal human kidney membrane. This gave an optical density of 1.0 ± 0.1 (standard deviation). The background was 0 ± 0.1 optical density units (O.D.) using medium without mouse monoclonal antibody. Wells that gave a reaction on the breast cancer membrane extract of greater than 0.7 O.D. were saved.

For the indirect immunofluorescence cell line assay 100,000 breast cancer cells of the immunizing cell line were placed overnight with appropriate media in each chamber of a set of eight chambered slides. Similarly, 100,000 fibroblast cells from cell line CC95 were incubated overnight in chambered slide wells. The cells were washed with PBS containing 1% BSA. The wells, both breast cancer and fibroblast, were incubated for 30 minutes at 4°C with 1:10 dilutions of hybridoma supernatant. The cells were again washed and incubated 30 minutes at 4°C with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-mouse Ig. The cells were washed three times, fixed in 1.5% formaldehyde in PBS for five minutes, and the chambers removed and rinsed in PBS. The slides were then mounted in a composition containing polyvinyl alcohol, glycerol, buffers and a preservative and examined with a fluorescence microscope. Hybridoma wells showing strong fluorescent binding to the breast cancer cells but no fluorescent binding to fibroblasts were saved. Five thousand one hundred fifty-six hybridoma wells revealed breast cancer reactivity in the initial screen.

Supernatants from the 5156 positive wells were then tested in solid phase ELISA with seven normal tissue membrane extracts (liver, lung, colon, stomach, kidney, tonsil, and spleen). Any well supernatant giving an ELISA O.D. greater than 0.3 was discarded. One thousand one hundred one of the supernatants were found to be unreactive with the normal tissue extracts.

The 1101 hybridoma supernatants were tested on frozen sections of human breast carcinoma tissues. Six micron sections were attached to slides, fixed 10 minutes in acetone at 4°C, dried 10 minutes at room temperature, washed with PBS, blocked with horse serum and incubated 20 minutes at room temperature with 100-200 μ l neat hybridoma supernatant. The slides were washed with PBS, and finally incubated 20 minutes at 37°C with a 1:50 dilution of peroxidase conjugated rabbit anti-mouse Ig, washed again with PBS, and finally incubated 7.5 minutes at 37°C with 0.5 mg/ml diaminobenzidine in 0.05 M Tris buffer pH 7.2 containing 0.01% hydrogen peroxide. The slides were stained with hematoxylin, dehydrated and mounted in a medium containing 35.9% methyl/n-butylmethacrylate copolymer, 7.1% butyl benzyl phthalate, and 0.3% 2,6-ditertiary-p-cresol. One hundred twenty-four wells yielded breast cancer selective binding and were cloned.

45

Purification and Class Determination

Immunoglobulin class and subclass of the monoclonal breast cancer selective antibodies were determined by an immunodot assay essentially the same as that described in McDougal et al., *J. Immunol. Meth.* 63:281-290 (1983). Antibodies were also internally labeled by growing 2-3 $\times 10^6$ hybridoma cells for four hours in methionine-free medium containing 0.2 μ Ci 35 S methionine. 35 S-labeled antibodies were immunoprecipitated with fixed *Staphylococcus A* cells, or with fixed *Staphylococcus A* cells precoated with rabbit anti-mouse immunoglobulin, and the immunoprecipitates were analyzed by SDS-PAGE to determine antibody light and heavy chain mobility, lack of extra chains, and the ability of each antibody to bind staphylococcal protein A.

0 256 714

The antibodies were expanded *in vivo*. Balb/c or F1 (C57B 6 x Balb/c) mice were primed with 0.5 ml. Bristane intraperitoneally (ip) and after 10-14 days inoculated with one million log phase hybridoma cells in PBS. Ascites fluid was stored at -70°C and thawed and filtered through a 0.8 micron filter unit before further purification.

Some IgG antibodies that bound staphylococcal protein A were purified by affinity chromatography on protein A-chromatographic resin containing either agarose, dextran and/or acrylamide with pH step gradient elution. IgG antibodies that did not bind protein A were precipitated by addition of ammonium sulfate to 40% saturation at 0°C or by binding to DEAE. Alternatively, IgG antibodies were purified by chromatography using a Sephadex S-200 column, followed by DEAE cellulose.

The precipitates were redissolved in PBS, dialysed to 20 mM Tris pH 7.2 and chromatographed on a 1.5 x 50 cm column of diethylaminoethyl cellulose (DEAE) eluting with a 1.5 liter 0-600 mM NaCl gradient at 4°C at a flow rate of 1 ml/min. In each case, column fractions were monitored by SDS-PAGE and the purest antibody fractions were pooled, concentrated to 1-3 mg/ml, dialysed to PBS/0.02% Na₂EDTA and stored at 4°C.

IgM antibodies were purified by gel filtration material on a 2.6 x 40 cm column of Sephadex S-300 or other gel filtration or resin containing agarose, dextran and/or acrylamide, eluting with PBS/0.01% sodium azide at room temperature at a flow rate of 1 ml/min.

22 Selectivity Determination

For evaluation of their selectivity for breast cancer, the purified antibodies were tested by immunoperoxidase section staining on sections of sixteen normal tissues, and by immunofluorescent cell sorting on live blood cell types. Immunoperoxidase staining was performed as above except that known dilutions of purified antibodies in PBS in the range of 1-40 µg/ml were used instead of hybridoma supernatants. The pure antibodies were first titrated to find the minimal concentration giving strong immunoperoxidase staining on breast cancer sections and then used at the concentration for the normal tissue tests. Peripheral blood cells (platelets, lymphocytes, red blood cells, granulocytes, and monocytes) were prepared by centrifugation using a medium which separates monocytes from polymorphonuclear leukocytes. The cells were reacted with antibody 2; the optimal concentration determined above for 30 minutes at 4°C, washed, reacted with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse Ig for 30 minutes at 4°C, washed again, and examined in a cell sorter. The wash buffer and diluents were PBS with 1% gelatin and 0.02% sodium azide. The cell sorter was equipped with a 76 micron nozzle and a one watt argon ion laser at 488 nm. An 80 mm confocal lens was used on the optical rail assembly for focusing. Other filters used were a 515 nm interference filter and a 515 nm absorbance filter (for scattered laser light) and a neutral density 1.5 filter for forward angle light scatter. Contour plots of log fluorescein fluorescence versus forward angle light scatter were used for sample analysis. No blood cell types showed detectable binding.

The binding behaviors of the preferred antibodies of the second class herein are reported in Table I below. The following abbreviations are used to denote structures bound by the antibodies: Ac, acini; G, glands; T, tubules; D, ducts; L, lumen; W, sweat glands; E, epithelium; S, sebaceous glands; Gr, granulocytes; Mk, megakaryocytes; M, macrophage; Ly, lymphocytes; Bl, basal layer; Fe, focal epithelium; A, alveolar lining cells; B, Bowman's capsule; Mu, muscle; and I, islets; H, hair follicles; U, glomeruli; and V, vessels/endothelial.

45

50

55

DERWENT PUBLICATIONS LTD.

0 256 714

MAB	Normal Tissue Bindings of MABs										Normal Breast				
	Esophagus	Lung	Kidney	Colon	Stomach	Breast	Tonsil	Liver	Heart	Ovary	Skin	Bone	Marrow	Uterus	Glandular
2G3	2E	2E	1A	2F	P	1L	0	1E	0	0	0	0	0	2L	2E
9C6	0	2E	0	0	0	1L	0	1Ly	2E	0	0	2Gr	0	0	2E
32A1	1H	1E	1A, M	1T, U	0	0	1Ly	0	0	0	1S, M	0	1M	0	0
33F8	0	2E	0	1I	0	0	1Ly	0	0	0	1M	1M	1E	0	0
35E10	0	2E	1A, 1M	0	0	1G	0	1Ly	0	0	2M	2Gr	0	0	1E
41B4	0	0	0	0	0	0	1E	0	0	0	1M	0	0	0	1E
87H7	1H	1E	0	0	1G	1G	0	1Ly, E	2	0	2H	0	0	0	2E
106A10	1Ac, D	1E	1M	0	0	0	2E	2M	0	0	2E, M	0	1E	0	2E
113F1	2Ac	2E	0	0	0	2G	0	1E	0	0	0	2L	0	0	0
120H7	0	1E	0	1I	0	1L	0	0	0	0	2E, 1S	0	0	0	0
140A7	1Ac, D	0	0	2I, R	0	0	1Ly	2	0	0	2E	0	0	0	0
200F9	1Ac	0	0	2L	0	0	0	0	0	0	2S	0	0	0	0
203F2	2Ac	1E	2A	2L	0	0	0	0	0	0	0	2H, M	1-2Gr	1G	0
219F3	1Ac	2E	0	1T	0	0	0	1Ly, E	0	0	2H, M	1-2Gr	1G	0	2E
245E7	1L	0	1A, M	0	0	2L	0	1E	0	0	2S	0	2L	1E	2L

1161

88051473

DERWENT PUBLICATIONS LTD.

0 256 714

55 56 57 58 59 60 61 62
TABLE I (Cont'd)

<u>MAB</u>	<u>P.I.F. CREAT</u>	Normal tissue Bindings of MAB's										<u>None</u> <u>Harmo</u> <u>Uterus</u> <u>Bladder</u> <u>Breast</u>	
		<u>Stomach</u>	<u>Colon</u>	<u>Intestine</u>	<u>Liver</u>	<u>Heart</u>	<u>Ovary</u>	<u>Skin</u>	<u>None</u>	<u>Harmo</u>	<u>Uterus</u>	<u>Bladder</u>	
1162	254H9	2E	1A	2I	0	16	0	0	0	0	0	0	2L
	260F9	1Ac	2E	0	1I	0	16	0	2E	2H	0	0	2E
	266B2	1Ac, 1D	2E	0	1I	0	0	0	2E, 2M	0	0	1E	1E
	317G5	1Ac	1	0	2I	16	0	0	2D	0	0	0	0
	369F10	0	0	0	0	16	0	0	0	0	0	0	0
	387H9	1D	0	0	0	0	0	0	1L, 1E, 1,	10	0	2	1E
	421E8	1Ac	1E	0	1I	0	16	0	1	0	0	0	0
	451C3	0	0	2A	0	0	1V	2L, 1H	0	0	2	16	0
	452E12	0	0	0	0	0	0	1	0	0	0	0	0
	452F2	0	0	0	0	0	0	0	0	0	0	0	0
	454A12	0	1M	0	16	0	0	0	0	1E, H	0	1E	1E
	454C11	1D	1-2E	0	1I	0	0	0	1E	1D	0	0	2
	457D7	0	0	0	0	0	16	0	1	0	0	0	0
	520C9	0	0	1I	0	0	0	0	0	0	0	0	0
	650E2	1Ac, 1	0	1-2A	2I	2I	0	0	20	0	0	0	2E

02051473

0 256 714

MAR	Pan- creas	Eso- phagus	Lung	Normal Tissue Bindings of MAMIS								Normal breast	
				Sto- mach	Colon	Brain	Tonsil	Liver	Heart	Ovary	Skin	Marrow	
69703	0	0	0	0	0	0	0	0	0	0	0	0	21
741F8	0	0	0	0	0	0	0	0	0	0	0	0	0
759E3	0	0	0	0	0	0	0	13	0	0	0	0	0
788G6	0	0	0	21	0	0	1Fe	0	0	0	0	0	0

0 = No binding
 1 = Moderate binding
 2 = Strong binding

0 256 714

Breast Cancer Cell Binding Range Determination

5 Antibodies were further evaluated for range of breast cancer cell line recognition by immunofluorescence assays on 14 breast cancer cell lines. Table II below reports the results of these tests for the preferred antibodies herein of the second class.

10

15

20

25

30

35

40

45

50

55

1164

14

00054479

0 256 714

MAR	MCF7	BR20	ZR751	MDA-MB231	CAM1	LAB	BT549	BT444	T47D	MDA-MB436	MDA-MB330	SKBR3	MDA-MB453	ZER7530
2G3	4	3	3	2	4	2	4	2	3	2	3	4	3	3
9C6	3	0	3	0	4	2	0	3	3	0	0	2	0	2
32A1	3	2	2	2	2	2	0	3	3	2	0	2	1	3
33F8	2	3	2	0	2	3	2	2	0	3	2	2	0	1
35E10	1	0	0	0	0	0	0	2	0	0	0	0	0	2
41B4	1	0	0	0	0	0	0	0	1	0	0	0	0	0
87H7	0	0	1	1	0	0	0	0	0	0	0	1	0	0
106A10	3	3	2	2	2	0	0	2	3	2	2	3	2	2
113F1	3	4	2	2	4	0	0	4	3	3	2	4	2	3
120H7	3	2	3	0	3	0	2	0	0	0	0	2	3	2
140A7	3	2	1	0	2	0	1	0	0	0	3	0	2	1
200F9	3	3	2	0	2	2	3	0	3	2	0	0	3	2
203E2	4	4	3	0	4	2	4	2	4	3	0	2	4	3
219F3	3	3	4	0	4	3	2	3	3	4	0	3	2	3
245E7	4	4	4	2	4	4	2	4	4	4	3	4	3	4

15
88051473

DERWENT PUBLICATIONS LTD.

0 256 714

TABLE II (Cont'd)

Breast Cancer Cell Line Binding of MAbs

MAB	4C7	9120	BR151	MDA-MB231	CAMAL	BR549	BR474	T470	MDA-MB157	MDA-MB330	SKBR3	MDA-MB436	ZR7530
254119	4	4	1	2	4	1	3	2	4	4	2	3	3
260F9	3	3	3	2	3	2	0	2	2	2	ND	4	2
266B2	1	2	2	2	3	0	0	0	2	2	2	2	2
31765	2	3	3	0	4	3	1	1	4	0	1	2	1
369F10	1	0	0	0	0	0	0	0	0	0	0	0	0
38719	3	2	2	3	3	0	0	2	2	2	2	2	2
421E8	2		2		2		0				0	0	0
451C3	1	2	2		2		2		4	2	2	0	2
452E12	0	0	0		0	0	0		0	0	0	0	0
452F2	0	1	2		2	2	0		1	0	0	3	2
454A12	2	?	2		2	3	2		3	2		2	2
454C11	?	2	2		2	2	1		1	0		4	2
457D7	0	0	0		1		0		0	0		0	0
520C9	1	0	1		2		0		1	0		3	2
650E2	1	2	3		3		0		1	1		1	3

1106

16

88051473

0 256 714

TABLE II (Cont'd)
Breast Cancer Cell Line Binding of MAbs

Mab	MG57	B120	ZR751	MDA-MB231	CAM1 ALAB	BT1549	BT474	T47D	MDA-MB157	MDA-MB130	SKBR3	BT483	MDA-MB1530	787530
69703	2			4		3		4	3		0	4	2	
741FH	1			2		0		2	0		4	2	2	
759LJ	0			2				2			3	0	4	
788G6	2			2				3			0	3	2	

0 = Negative 3 = Strong
 1 = Weak 4 = Very strong
 ? = Moderate

1167

22051473¹⁷

0 256 714

Non-Breast Cancer Binding of Monoclonal Antibodies

Finally, the antibodies were tested by immunoperoxidase staining on eleven non-breast malignancies. The results for the preferred antibodies herein for the second class are reported in Table III below. The numbers are the same as designated in Table I.

10

15

20

25

30

35

40

45

50

55

1168

18

0000000000

0 256 714

TABLE III
Nonbreast Cancer Bindings of MABs

MAB	Colon	Lung	Pan- creas	Pro- state	Uterus	Lym- phoma	Sto- mach	Eso- phagus	Bladder	Melano- ma	Ovarian
263	0	2	0	?	0	2	0	0	0	0	2
9C6	0	0	0	2	1	0	0	0	0	0	1
32A1	0	0	0	2	0	0	1	0	0	0	0
33F8	1	0	0	1	0	0	0	0	0	0	1
35E10	2	1	2	0	2	0	0	0	0	0	0
41B4	0	2	0	0	0	0	0	0	0	0	0
87H7	1	2	0	1	0	0	0	2	1	0	0
106A10	1	1	0	1	1	1	0	0	1	0	0
113F1	0	2	0	2	1	2	2	0	0	0	2
120H7	0	0	2	0	1	1	0	0	0	0	0
140A1	0	0	0	1	2	1	2	1	0	0	1
200F9	0	1	0	0	0	0	0	0	0	0	0
203E2									1	0	1
219F3	0	0	1	0	1	1	0	0	0	0	1
245E7	1	2	2	2	2	2	2	2	0	0	2

0 256 714

Nonbreast Cancer Bindings of Mice

TABLE III (Cont'd.)

	MAB	Colon	Lung	Prostate	Pancreas	Uterus	Lymphoma	Sarcoma	Melanoma	Bladder	Esophagus	Ovarian
254119							0	0	0	0	0	2
260F9	+	0	1	1	1	0	0	0	1	1	0	1
266R2	0	1	1	1	0	0	1	0	0	0	0	1
317G5	1	1	0	0	1	0	0	0	0	0	0	0
369F10	0	1	1	1	0	0	0	0	0	0	0	0
387H9							0	0	0	1	1	1
421E8	1	1	1	1	1	1	0	0	0	2	1	1
451C3	1	1	1	1	1	1	1	0	0	1	0	0
452E12	0	0	0	0	0	0	0	0	0	0	0	0
452F2	0	0	0	0	0	0	0	0	0	0	0	0
454A12	0	0	0	0	1	0	0	0	0	1	2	1
454C11	0	0	1	1	1	2	0	0	0	0	0	1
520C9	0	1	1	1	1	1	1	0	0	0	0	0
520D7	0	0	1	0	0	0	0	0	0	0	0	0
650E2	?	2	2	2	2	2	0	0	0	0	0	0

1170

CONT'D.

0 256 714

TABLE III (Cont'd)
Nonbreast Cancer Findings of Males

MAB	Colon	Lung	Prostate	Pancreas	Uterus	Lymphoma	Bladder	Stomach	Esophagus	Molar mouth	Ovarian
697M3	0	0	0	0	0	0	0	0	0	0	0
741F8	0	0	0	0	0	0	0	0	0	0	0
759E3	0	1	1	1	0	0	0	0	0	0	0
78866	1	2	0	0	0	0	0	0	0	0	2

1171

21

00051A73

0 256 714

The tumor:breast cancer range, breast:cancer cell binding range, and blood cell binding and selectivity characteristics for the monoclonal antibodies according to the invention are summarized in Table IV.

TABLE IV
MAB Candidates

MAB	Blood Cells	Tumor Range	Cell Range	Selectivity
263	0	1.00	1.00	0.078
9C6	0	0.86	0.57	0.063
32A1	0	0.33	0.79	0.078
33F8	0	0.19	0.71	0.063
35E10	0	0.52	0.14	0.070
4134	0	0.67	0.00	0.023
67H7	0	0.95	0.60	0.078
105A10	0	0.36	0.26	0.026
113F1	0	0.14	0.79	0.047
120H7	0	0.57	0.57	0.047
140A7	0	0.71	0.36	0.070
200F9	0	0.52	0.71	0.031
203E2	0		0.86	0.055
219F3	0	0.86	0.86	0.086
245E7	0	1.00	1.00	0.070
254H9	0		0.92	0.064
260F9	0	0.52	0.92	0.089
266B2	0	0.71	0.83	0.070
317G5	0	0.43	0.77	0.055
369F10	0	0.81	0.17	0.023
387H9	0	0.29	0.91	0.086
421E8	0	0.81	0.57	0.055
451C3	0	0.38	0.91	0.070
452E12	0	0.52	0.00	0.023
452F2	0	0.24	0.55	0.000
454A12	0	0.29	1.00	0.031
454C11	0	0.76	0.75	0.078
457D7	0	0.55	0.10	0.039
520C9	0	0.25	0.40	0.008
650E2	0	0.86	0.90	0.008
597B3	0	0.81	0.88	0.070
741F8	0	0.18	0.63	0.000
759E3	0	0.14	0.78	0.008
788G6	0	0.52	0.22	0.016

50 Antibody Affinity and Antigen Density

Several of the antibodies which may be employed herein were iodinated and tested for binding to MCF-7, CAMA1, SKBR3, or ZR7530 cells. The antibodies were labeled with ^{125}I using chloramine T to a specific activity of approximately 10 $\mu\text{Ci}/\mu\text{g}$. To determine immunoradiochemical purity, 100,000 cpm of two of the labeled antibodies in 0.5 ml fetal calf serum was serially absorbed with five aliquots of target cells for 15 minutes at 0°C (generally 4,000,000 cells per aliquot), and the remaining radioactivity in the supernatant after each absorption was determined.

0 256 714

For measurements of association constants, known concentrations of labeled and unlabeled monoclonal antibodies were incubated with target cells in fetal calf serum for 15 minutes in ice. Aliquots of the cell antibody mix were then counted in a gamma counter or filtered through Microfold filter plates (V & P Scientific) and the filters counted. To account for unbound antibody retained in liquid on the filters controls containing the same concentrations of antibody, but no cells, were done in parallel. Association constants and antigen copy number per target are calculated from the affinity test results and are reported in Table V below.

TABLE V
Affinity and Antigen Copy Number of MAbs

<u>Mab</u>	<u>n</u>	<u>K_a</u>	<u>Cell Line</u>
2G3	3700000	9.1×10^6	MCF7
9C5			
32A1			
33F8			
35E10			
41B4			
87H7			
105A10			
113F1	2300000	1.1×10^9	MCF7
120H7	210000	5.2×10^6	MCF7
140A7			
200F9			
203E2			
219F3			
245E7			
254H9			
260F9	30000	6.0×10^7	MCF7
266B2	80000	2.7×10^8	MCF7
317G5	3200000	1.5×10^6	CAMA1
369F10			
387H9			
421E8	400000	1.4×10^8	MCF7
451C3			
452E12	250000	6.8×10^6	SKBR3
452F2	470000	1.2×10^8	MCF7
454A12	390000	4.8×10^7	ZR7530
454C11			
457D7	500000	8.2×10^6	SKBR3
520C9			
650E2			
697B3			
741F8			
759E3			
788G6			

55

0 256 714

Characterization of Antigen Specificity

For identifying the antigens recognized by the monoclonal antibodies, immunoprecipitation of the antigen was carried out according to the following method. Eight mm diameter polystyrene balls (Precision Plastic Ball Co.) were covered with 10% fuming nitric acid in glacial acetic acid and were incubated for three hours in a 50°C water bath. Following the acid treatment, the balls were rinsed three times with distilled water, covered with 1% sodium dithionite in 0.1 M NaOH and incubated three hours in a 50°C water bath. The balls were again rinsed three times with distilled water, covered with 0.1% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), 0.2% suberic acid (suberic acid dissolved in dimethylformamide) and incubated overnight at room temperature. The balls were rinsed three times with distilled water, and marked for identification.

Purified monoclonal antibodies were diluted 0.2 mg/ml in 2-(N-morpholino) ethane sulfonic acid buffer, and the previously treated and marked polystyrene balls were placed in individual tubes and covered with 450 microliters diluted antibody and 50 microliters of fresh 1% EDAC. Tubes were capped and incubated at 25°C for 24 hours. Following this incubation, the balls were rinsed twice with PBS and were either used "fresh" or were stored for several days at 4°C before use.

Freshly labeled target cell extracts were prepared from human breast cancer cell lines labeled with 125-I by the lactoperoxidase method of Marchalonis, J., "An Enzymic Method for the Trace Iodination of Immunoglobulins and Other Proteins", *Biochem. J.* 113:299-305 (1969), or with 35-S by growth in 35-S methionine. The labeled cells were dissolved in solubilization buffer (1% (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5). Four parts of labeled extract were mixed in a vessel with one part solubilization buffer containing 50 mg/ml bovine serum albumin, to give a final concentration of 10 mg/ml SSA. The balls coated with monoclonal antibody were added to the vessel and were incubated four hours on ice with shaking. Labeled antigen was pipetted from the vessel and the balls were rinsed four times with solubilization buffer. The balls were then removed, placed in individual tubes with 100 microliter Laemmli SDS gel sample buffer, and were incubated three minutes in boiling water. The balls were removed and the samples were run on an SDS gel with appropriate standards.

Immunoprecipitation tests on the antibodies indicated that five of them (454C11, 452F2, 520C9, 741F8, and 759E3) all bind a monomeric protein of about 200 K daltons found in cancerous breast tissue. Two of the five (520C9 and 741F8) are believed to recognize the same epitope on the 200 K dalton protein. 454C11 and 759E3 bind a second epitope on the same antigen, and 452F2 binds a third epitope on the same antigen. Four of the antibodies (41B4, 87H7, 452E12, 457D7) bound to a 240 K daltons intracellular antigen. Seven antibodies (2G3, 200F9, 203E2, 245E7, 369F10, 697B3 and 788G6) bound to high molecular weight mucins (HMW). Two antibodies (451C3 and 454A12) bound to transferrin receptors in the form of a 95,000 dalton antigen. Neither 451C3 nor 454A12 blocked binding of transferrin to the receptor. The antigen binding characteristics of the monoclonal antibodies according to the invention are summarized in Table VI.

45

46

50

55

DERWENT PUBLICATIONS LTD.

0 256 714

TABLE VI

	<u>MAB</u>	<u>Antigen</u>
22	2G3	HMW Mucin
	9G6	70 K
	32A1	
	33F8	66 K
	35E10	80 K
	41B4	240 K
	87H7	240 K
	106A10	55 K a
	113F1	40, 60, 100, 200 K Very Diffuse
	120H7	HMW Mucin
	140A7	Glycolipid (pentasaccharide)
	230F9	HMW Mucin
	233E2	HMW Mucin
	219F3	
	245E7	HMW Mucin
	254H9	
26	260F9	55 K b
	266B2	55 K b
	317G5	42 K c
	369F10	HMW Mucin
	387H9	40 K
36	421E6	
	451C3	Transferrin receptor
	452E12	240 K
	452F2	200 K
	454A12	Transferrin receptor
35	454C11	200 K
	457E7	240 K
	520C9	200 K
	650E2	42 K c
	597B3	200 K
40	759E3	200 K
	732G6	HMW Mucin

45 a = Different epitope than that bound by 260F9 and 266B2

46 b = Different epitope than that bound by 106A10; both 260F9 and 266B2 appear to bind to same epitope

50 c = Cross block each other

Antibody Isotype55 Antibody isotype was determined as follows: A grid of 5-mm squares was lightly drawn in pencil on the nitrocellulose sheet; and 1-ml droplets of antiisotype sera (Litton Bionetics, Kensington, Maryland, rabbit antisera to mouse κ , λ , α , γ_1 , γ_2a , γ_2b , γ_3 , and μ chains) were applied so that each row of squares received one spot of each heavy and light chain reagent. The sheet was incubated one hour at room

0 256 714

temperature in a moist chamber, rinsed quickly in PBS-BSA containing 1% (w/v), and left overnight in PBS-BSA at 4°C. Strips were cut 2021 with a scissors and stored at 4°C in PBS-BSA containing 0.02% sodium azide. Alternatively, strips were air-dried and stored desiccated at 4°C. A series of small tubes was prepared containing 3 ml hybridoma culture supernatant or supernatant diluted with PBS-BSA. 1:10 dilutions were generally successful; and some supernatants can be diluted as much as 1:200. A nitrocellulose strip was incubated in each tube for one hour at room temperature. The strips were rinsed three times in PBS-BSA and incubated for one hour at room temperature in diluted rabbit anti-mouse-horseradish peroxidase. The strips were rinsed twice in PBS-BSA and twice in Tris buffer. The strips were placed in Tris buffer containing diaminobenzidine and hydrogen peroxide until sufficient color developed on the anti-isotype spots (usually 3-4 minutes). The antibody isotypes are indicated in Table VII.

TABLE VII

Isotype of MABs

	MAB	Isotype
20	2G3	G1
	9C6	M
	32A1	G1
	33F8	G1
	35E10	M
25	41B4	G1
	87H7	G1
	106A10	G1
	113F1	G3
	120H7	M
30	140A7	M
	200F9	G1
	203E2	G1
	219F3	G1
	245E7	G1
35	254H9	M
	250F9	G1
	256B2	G1
	317G5	G1
	369F10	M
40	387H9	G1
	421E8	G1
	451C3	G1
	452E12	G1
	452F2	G1
45	454A12	G1
	454C11	G2A
	457D7	G1
	520C9	G1
	650E2	G1
50	697B3	G1
	741F8	G1
	759E3	G1
	788G6	G1

55

0 256 714

B. IL-2

The recombinant IL-2 employed in this example is des-alanine-IL-2_{se125}. The amino acid sequence of this IL-2 differs from the amino acid sequence of native human IL-2 in that it lacks the initial alanine of the native molecule, and the cysteine at position 125 has been changed to serine. Samples of *E. coli* that produce this IL-2 have been deposited by Cetus Corporation in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. USA on September 26, 1983 under accession number 39.452 and on March 6, 1984 under accession number 39.626 under the provisions of the Budapest Treaty.

The IL-2 was processed and purified as described in the text and Figure 1 of copending U.S. Serial No. 715,152 filed March 21, 1985, the disclosure of which is incorporated herein by reference, except that the *in vitro* disulfide bond formation was carried out using cupric chloride, as described in U.S. Patent No. 4,572,798 rather than o-iodosobenzoate. When the IL-2 was recovered from the chromatography step(s) it was lyophilized and resuspended in a neutral aqueous buffer. The purity of the recombinant IL-2 after the chromatography step(s) was at least about 95%, and the IL-2 contained less than about 0.02 ng/ml endotoxin as determined by the Limulus amebocyte assay.

The purified IL-2 was formulated at a concentration of 0.3 mg/ml with 50 mg/ml mannitol.

C. Model

The intraperitoneal mouse tumor model OVCAR 3, a human-derived ovarian cell line from the National Institutes of Health (Dr. Hamilton) and available from the American Type Culture Collection, Rockville, MD is employed.

D. Subcutaneous Xenograft

Female immunocompetent mice (Charles River Laboratories, Inc., Wilmington, MA) are treated, 5 per group, subcutaneously with the OVCAR-3 model. Tumors are measured on days 0, 3, 10, 14 and 17.

E. Results

IL-2 alone, one of the breast cancer antibodies 280D11, 520C9, or 454A12 alone (identified in Table X below), and IL-2 in conjunction with the antibody may be injected intravenously into the mice on the first day after tumor implantation (Day 1) or on the third day after tumor implantation (Day 3) with the dose and schedule indicated in Table VIII.

40

45

50

55

DERWENT PUBLICATIONS LTD.

0 256 714

TABLE VIII

<u>Agent(s)</u>	<u>Dose/kg</u>	<u>Schedule</u>
PBS	-	Daily for 14 days- begin at Day 1 post- implant
IL-2 alone	$3.75-7.5 \times 10^6$ U/kg	Daily for 14 days-begin at Day 1
Monoclonal antibody 280D11, 520C9 or 454A12 alone	25-50 mg/kg	Once every 2 or 3 days for 14 days- begin at Day 1 or 3
IL-2 and 280D11, 520C9, or 454A12	$3.75-7.5 \times 10^6$ U/kg 25-50 mg/kg	Daily for 14 days- begin at Day 1 Once every 2 or 3 days for 14 days- begin at Day 1 or 3

The maximum tolerated dose of IL-2 was found to be between 50 and 100 KU of IL-2 given daily to nude mice for 14 days and 150-200 KU IL-2 given daily to immunocompetent mice for 14 days.

26 The administration of the combination of agents as provided in Table VIII is expected to reduce tumor growth greater than the administration of either agent alone, although no work has been actually done confirming such results.

27 When the experiments were repeated using only 10 KU of IL-2 and 500mg of 280D11 administered twice daily for 3 days at Day 4, no effectiveness was seen. The dosage and scheduling must be adjusted to obtain efficacious results, i.e., the maximum tolerated dose of each component must be determined and then a schedule of twice daily for at least 10 days must be followed. Each type of cancer and antibody require different dosages and schedules, to be determined by routine experimentation.

28 In an alternative scheduling to that given in Table VII, the maximum tolerated dose of IL-2 may be administered alone for one week daily intraperitoneally (ip) or intramuscularly (im) near the tumor, followed by administering one-half the maximum tolerated dose of IL-2 and the maximum tolerated dose of the antibody as separate intravenous boluses.

EXAMPLE II

A. Preparation of Ricin A Chain

45 A soluble recombinant ricin A which requires no solubilization to be subjected to purification and to display cytotoxicity was prepared as follows. When the coding sequence for ricin A was placed in direct reading frame with the DNA encoding leader sequence of phoA to form a putative fusion peptide, so that the leader sequence is the N-terminal portion of a leaderricin A chimera, the ricin A sequences so disposed result in the soluble cytotoxic material.

50 Expression vectors containing the genes for the precursor proteins contained in pRT3 (ATCC Deposit No. 67.027, deposited March 7, 1986), pRT17 (ATCC Deposit No. 67.026, deposited March 7, 1986), and pRT38 (ATCC Deposit No. 67.025, deposited March 7, 1986) or their mutagenized forms were constructed. Transforming host cells with these expression vectors resulted in solubilization of the precursor protein encoded. The arg-arg modified precursor was cleaved with trypsin; the A and B portions of the precursors were produced as separate proteins, as herein described.

55 In the phoA expression system, the essential component is the terminated phoA leader sequence upstream of, proximal to, and at 1 frame with the ricin A coding sequence, wherein the ricin A coding sequence is initiated by an ATG codon. The two coding sequences must be, of course, provided with a compatible bacterial promoter, which was the phoA promoter already associated with the leader. Additional-

0 256 714

city, production was improved in the presence of a positive retroregulator sequence which was the positive retroregulator sequences associated with the crystal protein of B. thuringiensis, which are described extensively in European Publication 174,725 published March 19, 1986. This was provided on bacterial transport vectors which included replicons and selectable markers.

The vectors were then used to transform a suitable prokaryotic host which was grown under conditions suitable for the particular host chosen, most frequently under conditions whereby the promoter placed in control of the expression system was suppressed.

The production of the toxin A was then induced by providing conditions which effect expression under control of the chosen promoter and the production permitted to proceed for sufficient time to effect a desired accumulation of product. The protein product was then isolated by disrupting the cells and the cellular debris was removed. The toxin A produced was then further purified using standard techniques known in the art as applied to freely soluble proteins. However, the efficiency of the extraction and purification was enhanced by treating partially clarified extract with phenyl sepharose. The solubility of the toxin A in the sonicate (once separated from the membrane or other associated materials) was shown by its ability to remain in the supernatant when the sonicate was subjected to centrifugation at high speed, 100,000 x g for 30 minutes, to spin down insoluble proteins.

A total of 2 ml of this soluble toxin A (at 9.0 mg/ml) was reduced by adding 2 μ l of fresh β -mercaptoethanol (to 1.1%) and incubating at room temperature overnight. The 2 ml of reduced toxin A was applied to a desalting column equilibrated with 0.1M sodium phosphate pH 8.0 followed by 0.5 ml of buffer to make 2.5 ml same application volume. The next 30 ml of eluate (buffer was applied) was collected as desalting toxin A.

B. Conjugation of Recombinant A to Antibody

A cell line producing an anti-bigast monoclonal antibody designated 520C9, described more fully above, was deposited as Accession No. HB8896 on January 9, 1985 in the American Type Culture Collection (ATCC), Rockville, MD. This antibody was reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) at room temperature and then chilled, and then sufficient 2-iminoisotiolane (IT) was added to give 2.5 IT molecules per antibody molecule.

A total of 166 μ l of propylene glycol was added to 6.24 ml of the IT-derivatized antibody. The 2.32 ml of toxin A chain described above was added to initiate the conjugation reaction. The mixture was incubated at room temperature for two hours.

The conjugation reaction mixture was applied to a sizing (gel filtration) HPLC column using an eluting buffer of 0.1M sodium phosphate, pH 8.0. A total of 78% recovery of the purified immunoconjugate was obtained from the column.

C. IL-2

The recombinant IL-2 employed in this example is des-alanine-IL-2₁₋₁₂₅. The amino acid sequence of this IL-2 differs from the amino acid sequence of native human IL-2 in that it lacks the initial alanine of the native molecule, and the cysteine at position 125 has been changed to serine. Samples of E. coli that produce this IL-2 have been deposited by Cetus Corporation in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. USA on September 26, 1983 under accession number 39.452 and on March 6, 1984 under accession number 39.626 under the provisions of the Budapest Treaty.

The IL-2 was processed and purified as described in the text and Figure 1 of U.S. Patent No. 4,604,377, except that the in vitro disulfide bond formation was carried out using cupric chloride, as described in U.S. Patent No. 4,572,798 rather than o-iodosobenzoate. When the IL-2 was recovered from the chromatography step(s) it was lyophilized and resuspended in a neutral aqueous buffer. The purity of the recombinant IL-2 after the chromatography step(s) was at least about 95% and the IL-2 contained less than about 0.02 ng/ml endotoxin as determined by the Limulus amebocyte assay.

The purified IL-2 was formulated at a concentration of 0.3 mg/ml with 50 mg/ml mannitol.

DERWENT PUBLICATIONS LTD.

0 256 714

D. Model

The target cells employed are murine tumor P388 leukemia cells, obtainable from the American Type Culture Collection, Rockville, MD.

E. Subcutaneous Tumor Injection

The tumor cells are harvested from culture suspensions, inoculated subcutaneously (sq) or intraperitoneally (ip) into the appropriate type of mouse.

F. Results

IL-2 alone, the immunotoxin described above alone, and IL-2 with the immunotoxin may be administered intraperitoneally to the mice, beginning one day after tumor implantation (Day 1), with the dose and schedule indicated in Table VIII.

TABLE VIII

	<u>Agent(s)</u>	<u>Dose/kg</u>	<u>Schedule</u>
25	PSS		
25	IL-2 alone	$3.75-7.5 \times 10^6$ U/kg	Daily for 14 days- begin at Day 1 post- implant
30	Immunotoxin alone	100 ug/kg	Daily for 7 days- begin at Day 1
35	IL-2 and Immunotoxin	$3.75-7.5 \times 10^6$ U/kg 100 ug/kg	Daily for 14 days- begin at Day 1 Daily for 7 days- begin at Day 1

The maximum tolerated dose of IL-2 was found to be between 50 and 100 KU of IL-2 given daily to nude mice for 14 days and 150-200 KU of IL-2 given daily to immunocompetent mice for 14 days. The administration of the combination of agents as provided in Table VIII is expected to reduce tumor growth greater than the administration of either agent alone.

In an alternative scheduling to that given in Table VIII, the maximum tolerated dose of IL-2 may be administered alone for one week daily ip or im near the tumor, followed by administering one-half the maximum tolerated dose of IL-2 and the maximum tolerated dose of the immunotoxin, as separate intravenous boluses. The dosage and scheduling must be adjusted to obtain efficacious results. Each type of cancer and immunotoxin will require different dosages and schedules, to be determined by routine experimentation.

G. EXAMPLE III

An immunotoxin (IMT) was constructed as described in Example II except that an anti-breast monoclonal antibody designated 260F9, described more fully above (deposited as ATCC No. HB-8488) was employed instead of 520C9. The resulting immunotoxin was diluted in saline and 0.01% mouse serum albumin.

The IL-2 employed was the same as Example II. The target cells employed were human breast carcinoma cells from a cell line designated Mx-1 obtained from the National Institutes of Health. The tumor cells were implanted sq into nude mice.

0 256 714

The scheduling and dosing were as follows. The immunotoxin was administered every other day for a total of six times intravenously at 3.5 µg 20g mouse and 7.0 µg 20g mouse. The IL-2 was administered daily for nine days intraperitoneally at 10 kilounits dose and 100 kilounits dose. Both started on day 0 (the seventh day after implantation of the tumor) and had overlapping schedules when administered concomitantly. The results are shown in Table IX below.

TABLE IX

<u>Group</u>	<u>ΔBW*</u>	<u>Deaths</u>	<u>ΔTV**</u>	<u>ΔT/C***</u>
<u>IL-2</u>				
10KU	1.03	0/5	15.0	76
100KU	1.14	1/5	9.3	47
<u>IMT</u>				
3.5µg	0.98	0/5	9.4	47
7.0µg	0.92	0/5	4.7	24
<u>Combination</u>				
10 KU IL-2/3.5 µg IMT	0.92	0/5	12.9	55
100 KU IL-2/3.5 µg IMT	0.95	1/5	11.3	57
10 KU IL-2/7.0 µg IMT	0.75	2/5	3.6	18
100 KU IL-2/7.0 µg IMT	0.85	2/5	0.9	4
<u>Saline Control</u>	1.09	0/5	19.3	100

*ΔBW is change in body weight as measured by the ratio of mean body weight (in g) at 14 days after treatment to mean body weight (in g) at the start of treatment.

**ΔTV is change in tumor volumes as measured by the ratio of mean tumor volume (in mm³) at 14 days after treatment to mean tumor volume (in mm³) at the start of treatment.

***ΔT/C is the ratio of treated tumor volumes to control tumor volumes. (e.g., ΔT/C = 40 means there was 60% tumor growth inhibition)

The results indicate that the combination was approximately additive with respect to its antitumor effects; toxicities were enhanced slightly. Alteration of the dose/route/schedule may alter the efficacy and toxicity results.

The monoclonal antibody-producing hybridomas listed below were deposited with the American Type Culture Collection (ATCC) or Invivo International Inc. (IVI) under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of the viable culture for 30 years from date of deposit. The hybridomas will be made available by ATCC or IVI under the terms of the Budapest Treaty, and subject to an agreement between the assignee of this application, Cetus Corporation, and ATCC or IVI which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws. The assignee has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable culture of the same cell line.

Each hybridoma designation listed in the left column of Table X corresponds to the monoclonal antibody producing the designated monoclonal antibody.

0 256 714

TABLE X

<u>Cell Line Designation</u>	<u>IVI Accession Number</u>
9C6	IVI-10056
41B4	IVI-10057
374J	IVI-10059
106A10	IVI-10060
120H7	IVI-10061
200F9	IVI-10062
254H9	IVI-10063
421E8	IVI-10064
32A1	IVI-10066
35E10	IVI-10057
140A7	IVI-10069
203E2	IVI-10070
219F3	IVI-10072
337H9	IVI-10073
452E12	IVI-10074
454A12	IVI-10075
457D7	IVI-10076
597B3	IVI-10077
741F8	IVI-10078
759E3	IVI-10079
788G6	IVI-10080
451C3	IVI-10081
452F2	IVI-10082
650E2	IVI-10083

<u>Cell Line Designation</u>	<u>ATCC Deposit Date</u>	<u>ATCC Accession Number</u>
260F9	1/27/84	HB-8488
2G3	1/27/84	HB-8491
33F8	1/9/85	HB-8697
113F1	1/27/84	HB-8499
245E7	1/27/84	HB-8489
256B2	1/27/84	HB-8486
317G5	1/27/84	HB-8485
369F10	12/13/84	HB-8682
454C11	1/27/84	HB-8484
280D11	1/27/84	HB-8487
520C9	1/8/85	HB-8696
*260F9-1C9	11/7/84	HB-8562

*This clone is a descendent of 260F9 and was found to be a better antibody producer than 260F9.

In summary, the present invention is seen to provide a combination therapy for cancer using an amount of anti-tumor monoclonal antibody and/or immunotoxin and IL-2 together that is pharmacologically effective.

Claims

55 1. The use of a combination of IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human cells and/or at least one immunotoxin that binds selectively to human tumor cells in providing a therapeutic or prophylactic treatment of tumor burden in a warm-blooded mammalian host.

0 256 714

2. The use of claim 1 in which the IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human tumor cells and/or at least one immunotoxin that binds selectively to human tumor cells are to be administered separately.

3. A composition suitable for parenteral or subcutaneous prophylactic treatment of tumor burden comprising a mixture in pharmacologically effective amounts, of IL-2 from a mammalian species and at least one immunotoxin that binds selectively to cells containing the tumor burden.

4. A composition suitable for parenteral or subcutaneous prophylactic treatment of tumor burden comprising a mixture, in pharmacologically effective amounts, of IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human tumor cells.

5. A composition according to claim 3 wherein the immunotoxin comprises an antibody that selectively binds to human breast and/or ovarian cancer cells and has a G or M isotype, and wherein the cancer being treated is breast and/or ovarian cancer.

6. A composition according to claim 3 or claim 4 wherein the immunotoxin comprises an antibody that is selected from the group consisting of: 250F9, 280D11, 245E7, 520C9, 113F1, 265B2, 454C11, 2G3, 33F8, 317G5, 369F10, 9C6, 35E10, 106A10, 367H9, 421E8, 451C3, 454A12, 650E2, 741F8, 759E3, and antibodies that are functionally equivalent to a member of said group and wherein the immunotoxin comprises a recombinant ricin A chain.

7. A composition according to any one of claims 3 to 6 wherein the IL-2 is human IL-2 and the composition further comprises a pharmaceutically acceptable carrier medium.

8. A composition according to any one of claims 3 to 7 wherein the IL-2 is mature human IL-2, des-alan-IL-2_{α1α2}, des-alan-IL-2_{α1α2α3α4α5α6}, IL-2_{α1α2α3}, IL-2_{α1α2α4α5}, des-alan-IL-2, or des-alan-IL-2_{α1α2α3}.

9. IL-2 from a mammalian species and at least one immunotoxin that binds selectively to human tumor cells for use in a method for therapeutic or prophylactic treatment of tumor burden in a warm-blooded mammalian host comprising administering to said host a pharmacologically effective amount of a combination of said IL-2, immunotoxin(s) and/or antibody(ies) optionally with the components of said combination to be administered separately to the host.

10. IL-2 and immunotoxin and/or antibody according to Claim 9 wherein the immunotoxin comprises a recombinant ricin A chain conjugated to a monoclonal antibody that binds selectively to human breast and/or ovarian cancer cells and has a G or M isotype, and wherein the tumor burden being treated is breast and/or ovarian cancer.

11. IL-2 and immunotoxin and/or antibody according to claim 9 or claim 10 wherein the antibody is 520C9 or 250F9 and the host is human.

12. IL-2 and immunotoxin and/or antibody according to any one of claims 9 to 11 wherein the combination is administered in repeated doses.

13. A process for the preparation of a composition suitable for parenteral or subcutaneous administration to mammalian hosts for therapeutic or prophylactic treatment of cancer comprising mixing together or formulating for such use IL-2 from a mammalian species and at least one monoclonal antibody that binds selectively to human tumor cells and/or at least one immunotoxin that binds selectively to human tumor cells.

45

50

55

1183

33

88051473